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Tumor necrosis factor-α stimulates fractalkine production by mesangial cells and regulates monocyte transmigration: Down-regulation by cAMP

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Tumor necrosis factor-α stimulates fractalkine production by mesangial cells and regulates monocyte transmigration: Down-regulation by cAMP.

Background. Fractalkine is a CX_3C chemokine for mononuclear cells that has been implicated in the recruitment and accumulation of monocytes seen in glomerular diseases. We investigated the mechanisms by which tumor necrosis factor (TNF)-α stimulates mesangial cell (MC) fractalkine expression, and the effects of MC-derived fractalkine on monocyte transmigration.

Methods. Cultured rat MCs were incubated with TNF-α, with or without pretreatment with pharmacologic inhibitors of protein kinases or transcriptional factors downstream to TNF-α. Fractalkine mRNA and protein were analyzed by Northern and Western blotting. Translocation of nuclear factor (NF)-κB was evaluated by immunocytochemical staining. Monocyte transmigration was determined by in vitro chemotaxis assay.

Results. TNF-α stimulated MC fractalkine mRNA as well as cell-bound and soluble protein expression in a dose- and time-dependent manner. The soluble fractalkine was shed from the cell-bound form via metalloproteinase-dependent cleavage, and mediated in part TNF-α–induced monocyte transmigration in vitro. The incubation of MCs with calphostin C [a selective inhibitor of protein kinase C (PKC)] or PD98059 [a selective inhibitor of p42/44 mitogen-activated protein kinase (MAPK) kinase] attenuated TNF-α–stimulated fractalkine mRNA and protein expression. Coincubation of MCs with calphostin C and PD98059 resulted in a synergistic inhibition of TNF-α–stimulated fractalkine mRNA and protein expression. Incubation of MCs with phorbol myristate acetate (PMA) for four hours resulted in an increase in fractalkine mRNA expression that could be suppressed by calphostin C or depletion of PKC by pretreatment with PMA for 24 hours. Further, activation of PKC-depleted MCs with TNF-α stimulated fractalkine mRNA expression that could be blocked by calphostin C. PD 98059, but not calphostin C, inhibited TNF-α–activated phospho-p42/44 MAPK and phospho-c-Jun levels, whereas only calphostin C inhibited TNF-α–activated phosphorylation of PKCζ/ι. The incubation of MCs with MG132, a NF-κB inhibitor, abolished TNF-α–induced degradation of inhibitory protein of NF-κB (I-κB)α, nuclear translocation of NF-κB, and fractalkine expression, without affecting phospho-c-Jun levels. In contrast, curcumin, an activating protein (AP)-1 inhibitor, attenuated TNF-α–stimulated phospho-c-Jun levels and fractalkine expression without discernible effects on TNF-α–induced degradation of I-κBα or NF-κB nuclear translocation. Neither PD 98059 nor calphostin C affected TNF-α–induced degradation of I-κBα or NF-κB nuclear translocation. Additional experiments examining the role of cAMP on MC fractalkine expression showed that the incubation of MCs with TNF-α and either db-cAMP or forskolin attenuated TNF-α–stimulated fractalkine mRNA and protein expression, preceded by attenuation of TNF-α–activated phosphorylation of p42/44 MAPK, and c-Jun, but not phosphorylation of PKCζ/ι or nuclear translocation of NF-κB.

Conclusion. The present data indicate that TNF-α activation of PKCζ/ι, p42/44 MAPK, c-Jun/AP-1, and p65/NF-κB are involved in TNF-α–stimulated MC fractalkine expression, with the soluble fractalkine mediating in part the TNF-α–induced monocyte transmigration in vitro. Uncoupling of p42/44 MAPK or c-Jun/AP-1 signals may contribute to cAMP inhibition of MC fractalkine expression activated by TNF-α.

Key words: cAMP, glomerular disease, chemokine, inflammation, fractalkine, crescentic glomerulonephritis, adhesion molecule, mesengial cells, cytokines, migration.

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Infiltration and accumulation of monocytes/macrophages within the glomerular mesangium are prominent pathobiologic features of human and experimental glomerular diseases. The mechanisms underlying monocyte infiltration involve a series of complex interactions between circulating leukocytes and resident glomerular cells. These interactions are intensified by the enhanced expression of adhesion molecules and chemokines by resident glomerular cells [1–3]. A key chemokine known to capture and direct migration of monocytes to sites of inflammation is fractalkine [4]. It is a novel CX_3C chemokine whose expression is regulated primarily by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β [4–6]. Unlike the CC or

CXC chemokines, which are secreted peptides by professional immune cells, fractalkine is presented on the surface of activated endothelial cells in the vasculature via its C-terminal transmembrane domain [4]. This cell membrane-bound fractalkine acts as an adhesive molecule and forms firm adhesion with circulating mononuclear cells expressing its cognate receptor, CX_3CR1 [7, 8]. When cleaved at a conserved motif proximal to the membrane by metalloproteinases, fractalkine can be shed and becomes a soluble chemoattractant [4, 9, 10]. Recent studies suggest potential pathophysiologic roles for fractalkine in rat and human crescentic glomerulonephritis, mouse cardiac allograft rejection, and human atherosclerotic coronary artery disease [11–14].

While fractalkine has been shown to be produced by non-endothelial cells, which include neurons and astrocytes in the central nervous system [5, 15, 16], dendritic cells within the tonsil and skin [6, 17], and epithelial cells in the gut [18], information is limited with regards to its expression by renal cells. The mesangial cells (MCs) are smooth muscle-like cells within the glomerulus that play a key role in maintaining glomerular capillary integrity and regulating glomerular filtration area [19]. In addition, MCs have been implicated in the recruitment of circulating leukocytes via, at least in part, synthesis of distinct sets of chemokines [20–24]. These distinct sets of MC-derived chemokines may facilitate extravasation and infiltration of leukocytes into the affected glomeruli, and augment the extent and severity of inflammation [25, 26]. TNF-α is a multifunctional cytokine that has been reported to induce the expression of an array of CC and CXC chemokine genes and their products in a variety of cell types including the MCs [24]. Recently, we found that cultured rat MCs produced fractalkine in response to TNF-α. In cultured endothelial cells, Garcia et al have shown that TNF-α induces fractalkine expression via a nuclear factor (NF)-κB–dependent mechanism [27]. However, they did not present additional intracellular mechanisms that could be responsible for fractalkine expression given the complexity of signaling pathways elicited by TNF-α [28–31]. Our present study investigated the mechanisms involved in TNF-α–stimulated fractalkine expression by MCs and the potential chemotactic effects of MC-derived fractalkine.

This report shows that TNF-α stimulates both cell-bound and cleaved soluble fractalkine production by MCs, and the soluble form mediates at least partially the TNF-α–induced monocyte transmigration in vitro. TNF-α activation of protein kinase C (PKC), p42/44 mitogen-activated protein kinase (MAPK), activating protein (AP)-1, and NF-κB are involved in TNF-α–stimulated MC fractalkine expression, and uncoupling of p42/44 MAPK or c-Jun/AP-1 signals may contribute to cyclic AMP (cAMP) inhibition of MC fractalkine production stimulated by TNF-α.

METHODS

Reagents

RPMI 1640 media, fetal calf serum (FCS), and other tissue culture reagents were obtained from Gibco BRL (Rockville, MD, USA). Culture flasks and plates were purchased from Costa Corning (Cambridge, MA, USA). Dibutyryl(db)-cAMP and goat IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Forskolin, calphostin C, phorbol myristate acetate (PMA), N-[2-bromocinnamyl (amino)ethyl]-5-isoquinolinesulfonamide (H-89), PD98059, SB203580, wortmannin, curcumin, MG132, quercetin, tosyl-phe-chloromethylketone (TPCK), pyrrolidine dithiocarbamate (PDTC), and GM 6001 were obtained from Calbiochem (La Jolla, CA, USA). Recombinant rat TNF-α, mouse fractalkine, and goat anti-rat fractalkine were obtained from R & D Systems (Minneapolis, MN, USA). Mouse anti-phospho-p42/44 MAPK was obtained from New England BioLab (Beverly, MA, USA). Mouse anti-phospho-c-Jun, anti-β-actin, and rabbit anti-phospho-PKCζ/ι, anti-p65/NF-κB, anti-inhibitory protein of NF-κB (I-κB)α were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). All chemicals used for total RNA isolation, Northern and Western blot analyses were of molecular grade and were obtained from Sigma or Roche Molecular Biochemicals (Mannheim, Germany) unless otherwise specified.

Cell cultures

Primary culturing of rat MCs was performed as described previously [32]. Cells were characterized as MCs on the basis of the presence of α-smooth muscle actin staining with the avidin-biotin-peroxidase method, using diaminobenzidine as the chromogen. Rat MCs between 10 and 20 passages were used and grown in RPMI 1640 media containing 10% FCS and 30 μg/mL of insulin. Murine macrophages (J774.A1) were obtained from the American Type Culture Collection (Rockville, MD, USA), and grown in Dulbecco's modified Eagle's medium (DMEM) according to the supplier's instructions.

Experiments were performed to determine the regulatory role of various protein kinases, NF-κB, AP-1 and cAMP on TNF-α–stimulated fractalkine expression by MCs. The effects of cAMP-dependent protein kinase (PKA), PKC, phosphatidylinositol 3-kinase (PI 3-K), p42/44 MAPK, and p38 MAPK on MC gene expression were evaluated by incubating cells with selective protein kinase inhibitors. Cells were first grown in RPMI 1640 containing 10% FCS until reaching 90% confluency, followed by RPMI 1640 + 0.5% FCS for 24 hours before pre-incubating with either a specific PKC inhibitor calphostin C (400 nmol/L for 1 h), a selective PKA inhibitor H-89 (2 μmol/L for 30 min), a p42/44 MAPK kinase inhibitor PD98059 (40 μmol/L for 30 min), a p38 MAPK inhibitor SB203580 (40 μmol/L for 30 min), or a PI 3-K in-

hibitor wortmannin (0.5 μmol/L for 30 min). After preincubation, cells were stimulated with TNF-α (5 ng/mL) for four or 24 hours at 37°C before harvesting for RNA or protein extraction. Further experiments were conducted to examine the role of AP-1 and NF-κB on TNF-α–stimulated fractalkine expression by MCs. The AP-1 inhibitor curcumin (20 to 40 μmol/L) and various NF-κB inhibitors (MG132 10 μmol/L, quercetin 50 μmol/L, TPCK 25 μmol/L, and PDTC 50 μmol/L) were preincubated with cells for 1.5 hours (curcumin, MG132 and quercetin) and one hour (for TPCK and PDTC). After preincubation, cells were stimulated with TNF-α 5 ng/mL for four or 24 hours at 37°C before harvesting for RNA or protein extraction. Additional studies were designed to examine the role of cAMP-mediated signaling pathways in MC fractalkine expression. Cyclic AMP-elevating substances, including db-cAMP (a cell permeable cAMP analog) and forskolin (an adenylate cyclase activator) were first incubated with MCs for four hours at 37°C to determine the role of cAMP on basal MC fractalkine mRNA expression. Further experiments were performed to examine the role of cAMP on TNF-α–stimulated MC fractalkine expression. Cells were preincubated with db-cAMP (0.2 to 2 mmol/L) for 15 minutes or forskolin (25 to 100 μmol/L) for 45 minutes, followed by TNF-α (5 ng/mL) stimulation for four or 24 hours at 37°C before harvesting for RNA or protein extraction.

RT-PCR

Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method [33]. Five micrograms of the RNA were reverse-transcribed (RT) to first strand cDNA using MMLV reverse transcriptase according to the manufacturer's instructions (Promega). Polymerase chain reaction (PCR) for rat and mouse CX_3CR1 (receptor for fractalkine) mRNA was performed by adding 3 μL (150 ng) of the first-strand cDNA in a total of 10 μL containing 50 mmol/L Tris, 0.25 μg/mL bovine serum albumin (BSA), 1 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 33 pmoles of each primer, and 0.5 U DNA polymerase. The primers for CX_3CR1, 5′-gctggaaggcataattacat-3′ (upstream), and 5′-ttgggactcataatggaaag-3′ (downstream), were based on a murine cDNA sequence (GenBank, AF102269), sharing 90% and 95% homology, respectively, with corresponding rat sequences (GenBank, U04808). The parameters for rapid cycle DNA amplifications were initial denaturation at 94°C for three minutes, then one second at 94°C, one second of annealing at 55°C, and 15 seconds at 72°C for 28 cycles (Idaho Technology), followed by a final extension at 72°C for 10 minutes. The amplified products were resolved in 5% polyacrylamide gels. The gels were stained with ethidium bromide and photographed.

Northern blot analysis

Ten micrograms of total RNA were electrophoresed on formaldehyde-denatured 1% agarose gels and subsequently transferred to nylon membranes according to standard protocols [34]. To synthesize fractalkine riboprobe, the cDNA fragment of rat fractalkine was first amplified by RT-PCR from glomerular RNA of nephritic rats, using the upstream primer, 5′-attttccaagacagaggacc-3′, and downstream primer, 5′-gaagagtagaccaagaaagg-3′ [5]. The digoxigenin-conjugated fractalkine riboprobe was then synthesized by in vitro transcription as described previously [35]. After hybridization, the blots were developed using CSPD® (Roche) as the substrate for alkaline phosphatase. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

Western blot analysis

To detect cell-bound fractalkine protein, MCs were washed and lysed in RIPA buffer containing 1% IGEPAL CA-630 and 0.25% deoxycholate (Sigma). Forty micrograms of cell lysate were heated at 100°C for 10 minutes, applied to 7.5% (for fractalkine) or 10% (for phospho-p42/44 MAPK, phospho-PKCζ/ι, phospho-c-Jun, IκBα, and β-actin) sodium dodecyl sulfate (SDS)-polyacrylamide gels, and electrophoresed. For detection of soluble fractalkine in the conditioned medium of TNF-α–activated MCs, media were concentrated with Centricon-10® (Millipore, Bedford, MA, USA), and 50 μg of protein were electrophoresed on 7.5% SDS-polyacrylamide gels. A prestained marker was also electrophoresed as a molecular weight marker. After electrophoresis, proteins were transferred onto a polyvinylidine difluoride (PVDF) membrane (Millipore) using a transblot chamber with Tris buffer. Western blots were incubated at 4°C overnight with primary antibodies. The next morning, membranes were washed with PBS/Tween-20 at room temperature for 40 minutes, and incubated with peroxidase-conjugated second antibodies at room temperature for one hour. After washing, the membranes were incubated with Renaissance® (NEN™ Life Science, Boston, MA, USA) according to the manufacturer's instructions.

Immunocytochemistry

For demonstration of p65/NF-κB nuclear translocation, MCs were incubated with TNF-α (5 ng/mL), or vehicles for 7.5, 15, or 30 minutes before fixation with 4% paraformaldehyde for one hour at 4°C. The cells were then washed by 1× phosphate-buffered saline (PBS)/0.2% TritonX-100 for 15 minutes and incubated with rabbit anti-p65/NF-κB at 4°C overnight. The next day, after washing for 15 minutes, the cells were incubated with biotin-conjugated anti-rabbit IgG at room tempera-

ture for one hour. Then, the cells were washed and incubated with the avidin-biotin-peroxidase reagent (Dakopatts, Glostrup, Denmark) at room temperature for one hour. After washing, immunodetection for p65/NF-κB was performed by adding 3-amino-9-ethylcarbazole chromogen as substrate according to the manufacturer's instructions.

Nuclear protein extraction and electrophoretic mobility shift analysis (EMSA)

Nuclear extracts from stimulated or non-stimulated MCs were prepared by cell lysis followed by nuclear lysis. Briefly, cells were suspended in 30 μL of buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol (DTT), and 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF); vigorously vortexed for 15 seconds; allowed to stand at 4°C for 10 minutes; and centrifuged at 2000 rpm for two minutes. The pelleted nuclei were resuspended in buffer containing 20 mmol/L HEPES (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L DTT, and 0.2 mmol/L PMSF for 20 minutes on ice, and then lysates were centrifuged at 15,000 rpm for two minutes. The supernatants containing the solubilized nuclear proteins were stored at −70°C until used for EMSA. The subsequent binding reaction was carried out by incubating 15 μg of nuclear proteins with 20,000 dpm of ^{32}P-labeled DNA probe which contained a consensus NF-κB recognition element in 20 μL of buffer [10 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 4% Ficoll, 1 mmol/L DTT, 75 mmol/L KCl, and 0.25 μg poly(dI-dC)] for 20 minutes prior to electrophoresis on a 5% non-denaturing polyacrylamide gel in a Tris-borate-EDTA buffer system. Dried gels were exposed to Kodak XR5 film for 4 to 20 hour at −70°C. Identification of the nuclear proteins that reacted with this probe was obtained by pre-incubating the nuclear extracts for five minutes with an antibody (2.5 μg) to the p65 subunit of NF-κB (Santa Cruz) before a radiolabeled probe was added. EMSA was then performed as outlined and the effect of the antibody on the NF-κB band examined.

In vitro chemotaxis assay

Medium obtained from TNF-α (5 ng/mL for 24 h)-activated MCs were placed in the lower compartment of Transwell® cluster plates (Costa Corning) with two compartment chambers separated by a 5-μm polycarbonate membrane filter. Murine monocyte/macrophages (J774.A1, 5×10^4 cells) were added to the upper chamber and incubated at 37°C for four hours in a humidified 5% CO_2 incubator. After incubation, the membrane filter was removed, stained with hematoxylin solution and mounted on a glass slide. The migrated monocytes were counted per ×200 high power field (HPF) with the use of a Nikon UFX-II microscope. Five randomly chosen HPFs were counted per membrane. Similar chemotaxis assay was performed with control MC conditioned media and compared with the results obtained by utilizing TNF-α–activated MC conditioned media. A goat anti-fractalkine antibody or goat IgG was used in the chemotaxis assay media to determine the biological specificity of fractalkine produced by MCs to stimulate monocyte transmigration. Additionally, to demonstrate that J774.A1 cells can be attracted by fractalkine, a separate set of experiments was performed in which different concentrations of recombinant murine fractalkine in 0.1% RPMI were placed in the lower chamber of Transwell® cluster plates. After incubation at 37°C for four hours, the monocytes migrated from the upper chamber were counted as described above, and compared to the results obtained by utilizing fractalkine-free control media.

Statistics

Data are expressed as mean ± SEM. All comparisons were done by analysis of variance (ANOVA) followed by Dunnett's *t* test using the StatView® package for the Macintosh computer (Abacus Concepts, CA, USA). A probability value of less than 0.05 was considered statistically significant.

RESULTS

Effects of TNF-α on fractalkine mRNA and protein expression by MCs

Rat MCs were incubated with different concentrations of TNF-α (0.5 to 50 ng/mL) for varying periods (2 to 24 h). The Northern and Western blotting showed that at basal state MCs expressed a low level of a single species of ~3.8 kb fractalkine mRNA and ~90 kD cell-bound fractalkine protein. Exogenous TNF-α stimulated MC fractalkine mRNA and protein expression in a concentration-dependent manner, and the maximal effect of TNF-α on MC fractalkine mRNA and protein expression was observed at 24 hours (Fig. 1).

MC-derived soluble fractalkine is shed from cell-bound fractalkine via metalloproteinase-dependent cleavage

Fractalkine has been reported to exist in both a membrane-bound and a cleaved soluble form in endothelial cells and neurons [4, 5]. Our Western blotting of TNF-α–activated MC conditioned media disclosed an approximately 75 kD soluble fractalkine fragment in both time- and dose-dependent manners (Fig. 1B). Because the cell lysate of TNF-α-stimulated MCs also contained a smaller (~75 kD) fragment of fractalkine (Fig. 1B), it was unclear whether the soluble fractalkine in the conditioned media was generated within the cells and subsequently secreted, or cleaved and shed from membrane-bound fractalkine. By using a broad-spectrum zinc-dependent

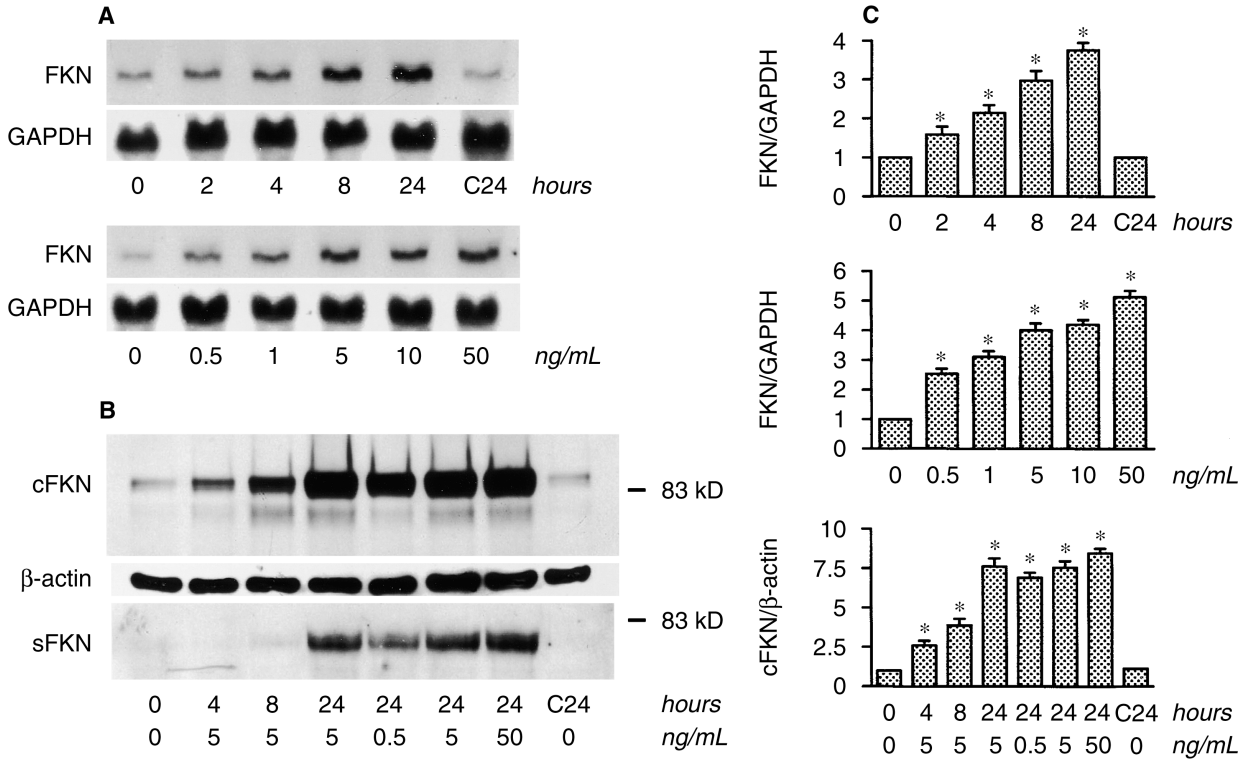

Fig. 1. Time-course and dose-response of TNF-α stimulation on fractalkine mRNA and protein expression. MCs were incubated with TNF-α (0.5 to 50 ng/mL) for the given time period (2 to 24 hours). Ten micrograms of total RNA, 40 μg of cell lysate, and 50 μg of concentrated condition media were analyzed for fractalkine mRNA and protein expression as described in the **Methods** section. (*A*) Representative Northern blots. Abbreviations are: C24, control at 24 hours; FKN, fractalkine mRNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNA. (*B*) Representative Western blots. Abbreviations are: cFKN, cell-bound fractalkine in cell lysate; sFKN, soluble fractalkine in concentrated conditioned media. (*C*) The intensity of densitometer readings corrected for GAPDH or β-actin, and relative to that of control. Values are mean ± SEM of four experiments. $*P < 0.05$ vs. control.

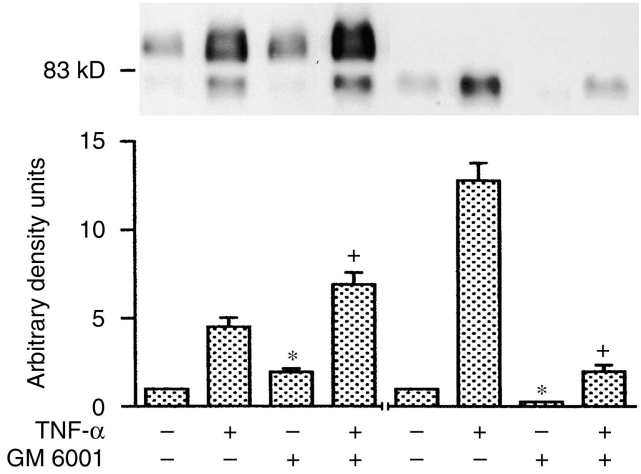

Fig. 2. Effects of GM 6001 on TNF-α–stimulated cell-bound and soluble fractalkine expression. MCs were incubated with TNF-α (5 ng/mL) for 24 hours, with or without pretreatment with GM 6001 (2 μmol/L) for 30 minutes. Upper panel shows representative Western blots. Lanes 1–4, cFKN in cell lysate; lanes 5–8, sFKN in concentrated conditioned media. Lower panel shows quantitative results of cFKN (corrected for β-actin) and sFKN relative to that of control. Values are mean ± SEM of four experiments. $*P < 0.05$ vs. control; $+P < 0.05$ vs. TNF-α–treated cells.

metalloproteinase inhibitor, GM 6001, we showed that the levels of soluble fractalkine were reduced in association with a corresponding increase in the amount of cell-bound fractalkine (Fig. 2). These results suggest that MC-derived soluble fractalkine was generated mainly from cleavage of its cell-bound form.

Chemotactic effects of MC-derived soluble fractalkine on monocyte transmigration in vitro

The monocytes used in the present study, J774.A1 cells, were found to constitutively express mRNA for CX_3CR1 and migrate in response to exogenous murine fractalkine in a dose-dependent manner (Fig. 3A). Furthermore, TNF-α (5 ng/mL for 24 h)-activated MC conditioned media recruited J774.A1 cells about fourfold over basal control. When the media was preincubated with different concentrations of an anti-fractalkine neutralizing antibody for 30 minutes at room temperature, monocyte migration was attenuated dose-dependently, with a maximal inhibitory effect of approximately 55%. In contrast, preincubating the media with the same concentrations of goat IgG did not affect monocyte transmigration (Fig. 3B).

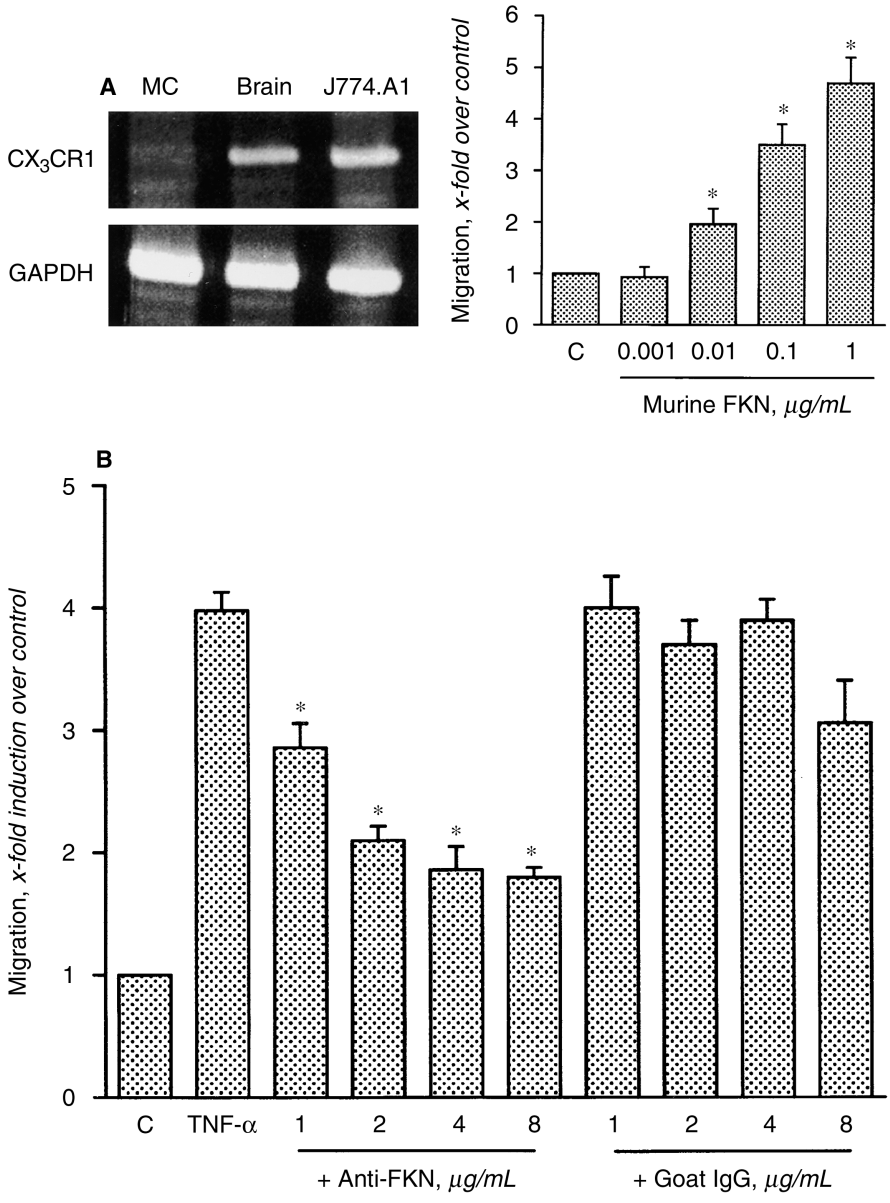

Fig. 3. Cleaved soluble fractalkine in the conditioned media of TNF-α-activated MCs mediates transmigration of J774.A1 cells. (*A*) Left. Representative RT-PCR showing constitutive expression of mRNA for fractalkine receptor (CX_3CR1) in J774.A1 cells, but not rat MC. Normal rat brain tissue was included as a positive control. Right. Exogenous murine fractalkine dose-dependently increases transmigration of J774.A1 cells using an in vitro chemotaxis assay as described in the **Methods** section. Data are mean ± SEM based on three separate experiments done in duplicate. $*P < 0.05$ vs. control (C). (*B*) Effects of the conditioned media obtained by incubating MCs with TNF-α (5 ng/mL) for 24 hours on monocyte transmigration. Similar studies were performed in the presence of different concentrations of a goat anti-fractalkine antibody or goat IgG. Data are mean ± SEM based on three separate experiments done in duplicate. $*P < 0.05$ vs. TNF-α alone.

Intracellular signaling of TNF-α–stimulated MC fractalkine expression

By using specific pharmacologic inhibitors, we first evaluated the role of various protein kinases (PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK) in TNF-α stimulated MC fractalkine expression. The incubation of MCs with calphostin C (400 nmol/L) or PD98059 (40 μmol/L) attenuated MC fractalkine mRNA and protein expression driven by TNF-α (Fig. 4 A, B). When calphostin C and PD98059 were coincubated with MCs, TNF-α–stimulated fractalkine mRNA and protein expression was inhibited synergistically (Fig. 4C). In contrast, H-89 (2 μmol/L), wortmannin (0.5 μmol/L) and SB203580 (40 μmol/L) did not have discernible effects on TNF-α–stimulated fractalkine expression (Fig. 4 A, B). Further, to explore the regulatory role of PKC isotypes on TNF-α–stimulated fractalkine expression, cells were incubated with an activator of the classical/novel isotype of PKC, PMA (0.1 μmol/L), for four hours. This resulted in an increase in fractalkine mRNA expression that could be suppressed by calphostin C (400 nmol/L). However, when MCs were pretreated with PMA (2 μmol/L) for 24 hours to deplete classical/novel PKC activity, the subsequent addition of PMA (0.1 μmol/L for 4 h) could not activate fractalkine gene expression (Fig. 5). In contrast, incubation of PMA-pretreated cells with TNF-α (5 ng/mL) for four hours still could up-regulate fractalkine mRNA to nearly the same levels as that seen in non-PMA-pretreated cells. This TNF-α–activated fractalkine mRNA expression in PMA-pretreated cells could be inhibited by calphostin C (400 nmol/L; Fig. 5).

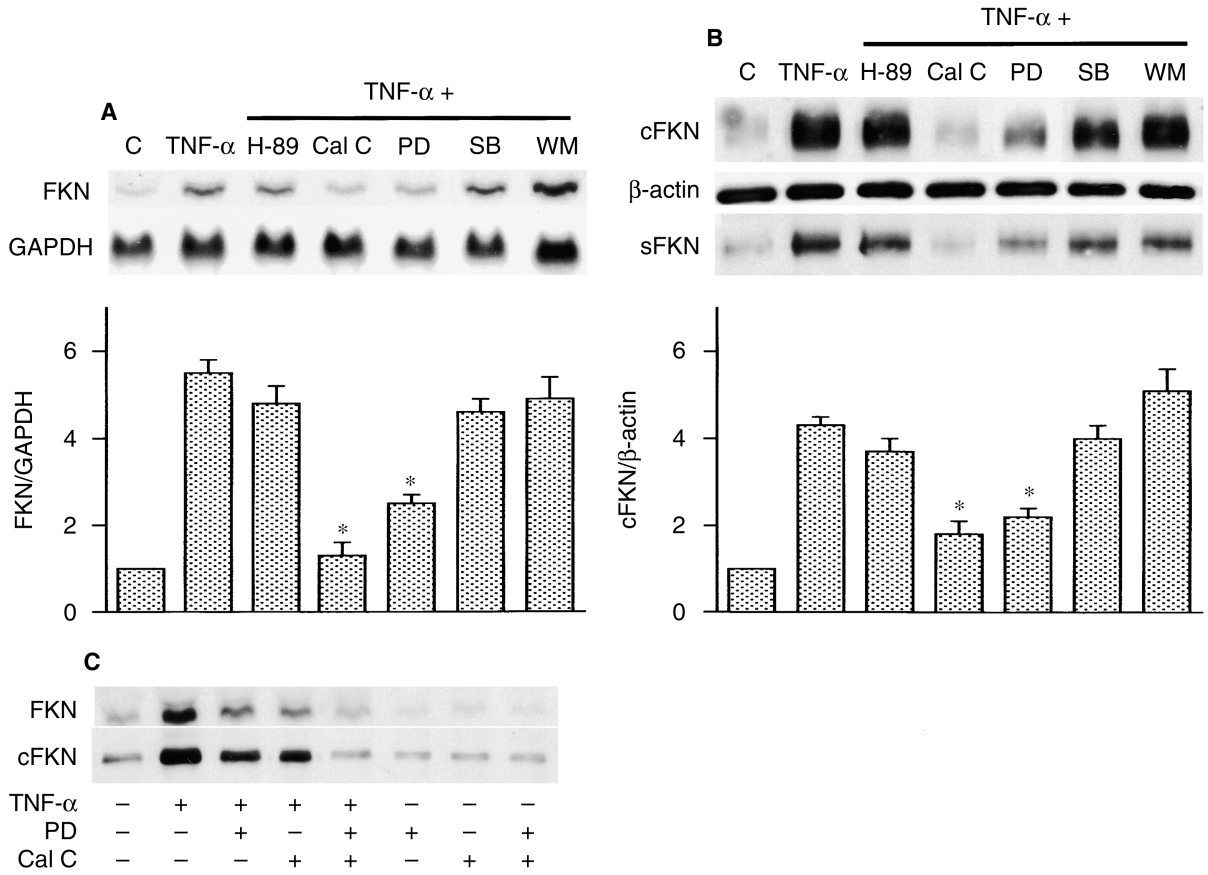

Fig. 4. Effects of protein kinase inhibitors on TNF-α–stimulated fractalkine mRNA and protein expression. MCs were incubated with TNF-α (5 ng/mL) for 4 or 24 hours, with or without pretreatment with the given pharmacologic inhibitor. (*A*) Upper panel shows representative Northern blots for H89 (2 μmol/L), Cal C (calphostin C 400 nmol/L), PD (PD98059 40 μmol/L), SB (SB203580 40 μmol/L), and WM (wortmannin 0.5 μmol/L). Lower panel shows quantitative results corrected for GAPDH, and relative to that of control. Values are mean ± SEM of four experiments. $^*P < 0.05$ vs. TNF-α–treated cells. (*B*) Upper panel shows representative Western blots. Lower panel shows quantitative results corrected for β-actin, and relative to that of control. Values are mean ± SEM of four experiments. $^*P < 0.05$ vs. TNF-α–treated cells. (*C*) Northern and Western blots showing that PD98059 and calphostin C synergistically inhibit expression of FKN and cFKN. Results are representative of four separate experiments.

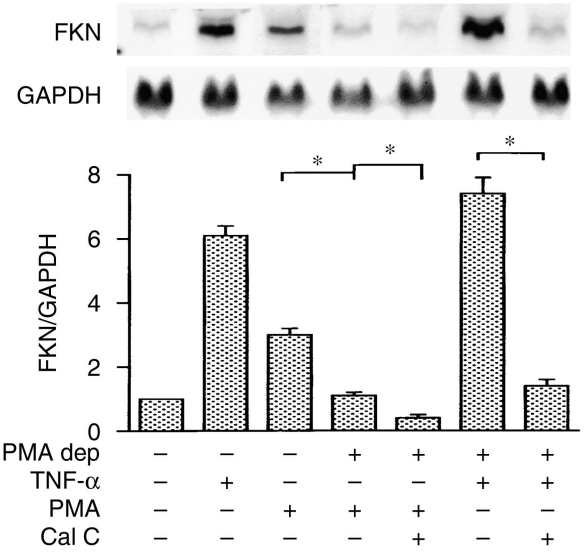

Fig. 5. Role of PKC in basal and TNF-α–stimulated fractalkine mRNA expression. Upper panel shows representative Northern blots. Lower panel shows quantitative results of fractalkine/GAPDH mRNA ratios relative to that of control. Abbreviations are: PMA dep, MCs were preincubated with PMA (2 μmol/L) for 24 hours to deplete endogenous PKC activity before stimulation with PMA (100 nmol/L) or TNF-α (5 ng/mL) for four hours; Cal C, MCs were pretreated with calphostin C (400 nmol/L) for one hour before stimulation with PMA or TNF-α for four hours. Values are mean ± SEM of four experiments. $^*P < 0.05$.

We next incubated the cells with various NFκ-B inhibitors such as MG132 (10 μmol/L), curcumin (40 μmol/L), quercetin (50 μmol/L), TPCK (25 μmol/L), and PDTC (50 μmol/L) before TNF-α stimulation. The results showed that TNF-α–stimulated fractalkine mRNA and protein expression was completely inhibited (Fig. 6). At the given concentrations, MG132 and curcumin were able to block TNF-α–induced degradation of I-κBα, nuclear translocation of p65/NF-κB, and p65/NF-κB binding activity (Figs. 7C, 8, and 9). However, unlike MG132, curcumin also is known as a c-Jun/AP-1 inhibitor [36, 37]. In this study, we found that curcumin at 20 μmol/L atten-

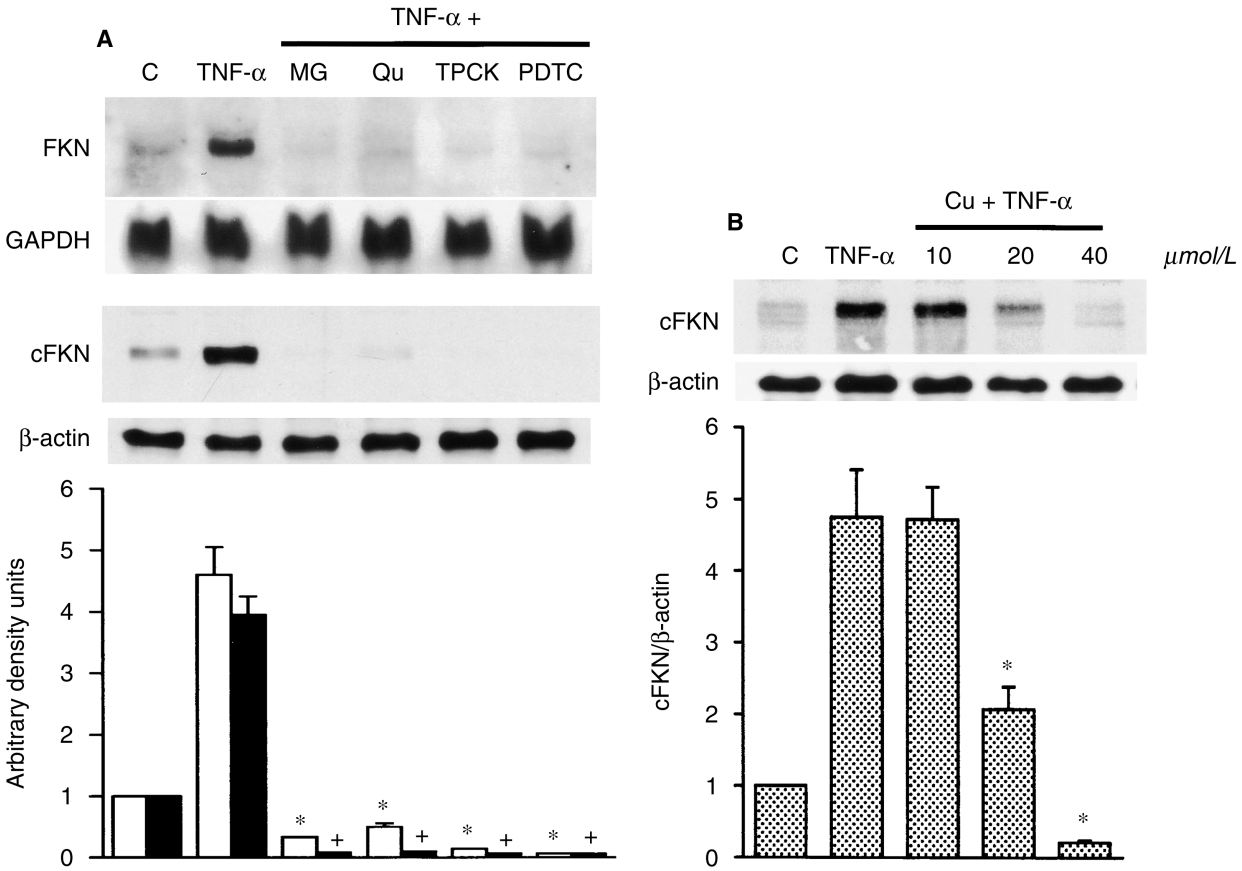

Fig. 6. Effects of NF-κB inhibitors and curcumin on TNF-α–stimulated fractalkine mRNA and protein expression. MCs were pretreated with the given NF-κB inhibitor or curcumin for 1.5 hours, followed by TNF-α (5 ng/mL) for 4 or 24 hours. (*A*) Upper panels show representative Northern (FKN) and Western (cFKN) blots. Abbreviations and quantities are: MG, MG132 (10 μmol/L); Qu, quercetin (50 μmol/L); TPCK (25 μmol/L); and PDTC (50 μmol/L). Lower panel shows quantitative results of fractalkine/GAPDH mRNA (□) and cFKN/β-actin (■) ratios relative to that of control. Values are mean ± SEM of four experiments. * and + denote $P < 0.05$ versus TNF-α–treated cells. (*B*) Upper panel shows representative Western blots for cFKN. Cu is curcumin (10 to 40 μmol/L). Lower panel shows quantitative results of cFKN/β-actin ratios relative to that of control. Values are mean ± SEM of four experiments. *$P < 0.05$ vs. TNF-α–treated cells.

uated TNF-α–activated phospho-c-Jun levels (Fig. 7A), and partially suppressed TNF-α–stimulated fractalkine protein expression (Fig. 6B), without affecting degradation of I-κBα (Fig. 7C) or nuclear translocation of p65/NF-κB (data not shown). Neither curcumin nor MG132 affected TNF-α–activated phosphorylation of p42/44 MAPK or PKCζ/ι (Fig. 7 B, D). Additional experiments were performed to examine the effects of PD98059 and calphostin C on TNF-α–activated signaling cascades. Our results indicated that PD 98059, but not calphostin C, inhibited TNF-α–activated phospho-p42/44 MAPK and phospho-c-Jun levels (Fig. 7 A, B), whereas only calphostin C inhibited TNF-α–activated phosphorylation of PKCζ/ι (Fig. 7D). Neither PD98059 nor calphostin C affected TNF-α–induced degradation of I-κBα (Fig. 7C) or nuclear translocation of p65/NF-κB (Fig. 8).

Effects of cAMP-elevating agents on TNF-α–stimulated MC fractalkine expression

At the basal state, incubation of MCs with db-cAMP (2 mmol/L) or forskolin (50 μmol/L) for four hours had no discernible effect on MC fractalkine mRNA expression (data not shown). In contrast, the preincubation of MCs with either cAMP-elevating agent dose-dependently attenuated TNF-α–stimulated fractalkine mRNA and soluble protein expression (Fig. 10). To explore the underlying mechanisms, MCs were incubated with TNF-α (5 ng/mL) and db-cAMP (2 mmol/L) for 7.5 to 30 minutes. The results showed that db-cAMP decreased TNF-α–activated phospho-p42/44 MAPK and phospho-c-Jun levels (Fig. 7 A, B), but not degradation of I-κBα (Fig. 7C), phosphorylation of PKCζ/ι (Fig. 7D), or nuclear translocation of p65/NF-κB (data not shown). Preincubation of MCs with forskolin also led to a similar downregulation of TNF-α–induced phospho-p42/44 MAPK and phospho-c-Jun levels, without alterations in I-κBα degradation or PKCζ/ι phosphorylation (data not shown).

DISCUSSION

This study demonstrated that cultured rat MCs at basal state expressed a low level of fractalkine mRNA and cell-

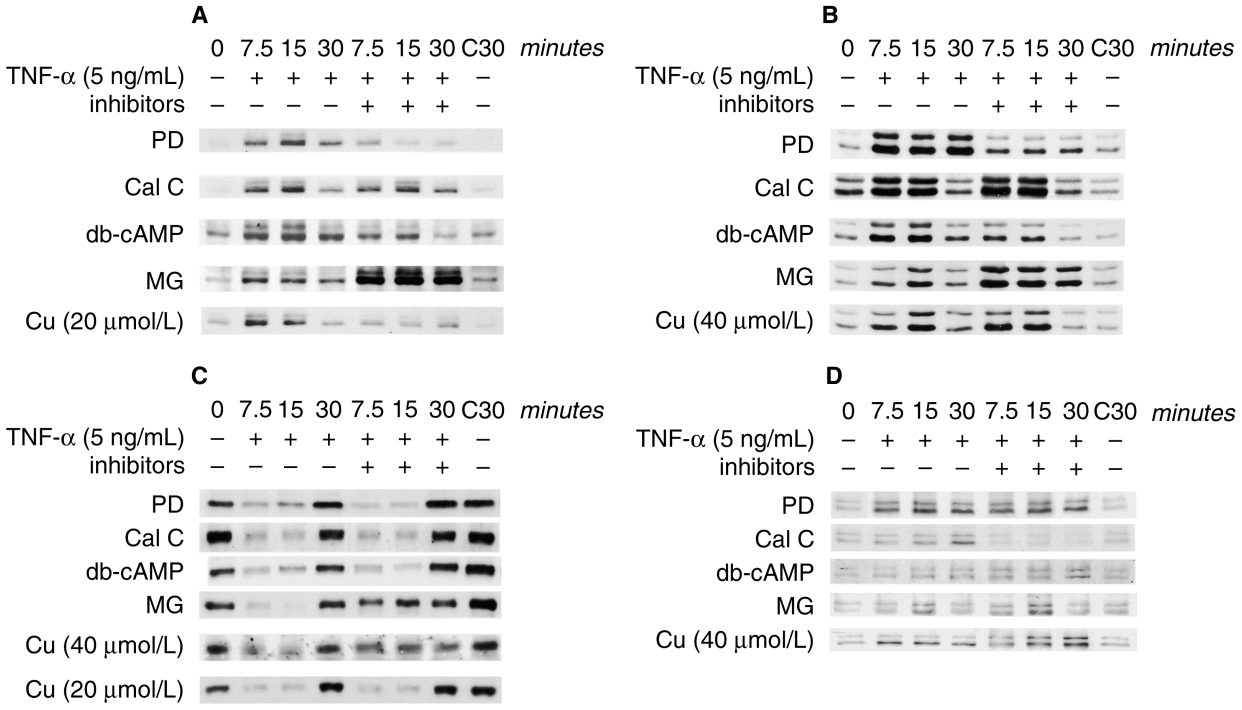

Fig. 7. Western blotting of phospho-c-Jun, phospho-p42/44 MAPK, I-κBα, and phospho-PKCζ/ι levels stimulated by TNF-α; effects of PD98059, calphostin C, db-cAMP, MG132, and curcumin. MCs were incubated with TNF-α (5 ng/mL) for 7.5, 15, and 30 minutes, with or without pretreatment with the given pharmacologic inhibitor. (*A*) Representative Western blots detected with an anti-phospho-c-Jun antibody. (*B*) Representative Western blots detected with an anti-phospho-p42/44 MAPK antibody. (*C*) Representative Western blots detected with an anti-I-κBα antibody. (*D*) Representative Western blots detected with an anti-phospho-PKCζ/ι antibody. These experiments were performed three times and similar results were obtained. Abbreviations are: PD, PD98059 (40 μmol/L); Cal C, calphostin C (400 nmol/L); db-cAMP (2 mmol/L); MG, MG132 (10 μmol/L); Cu, curcumin (20 or 40 μmol/L).

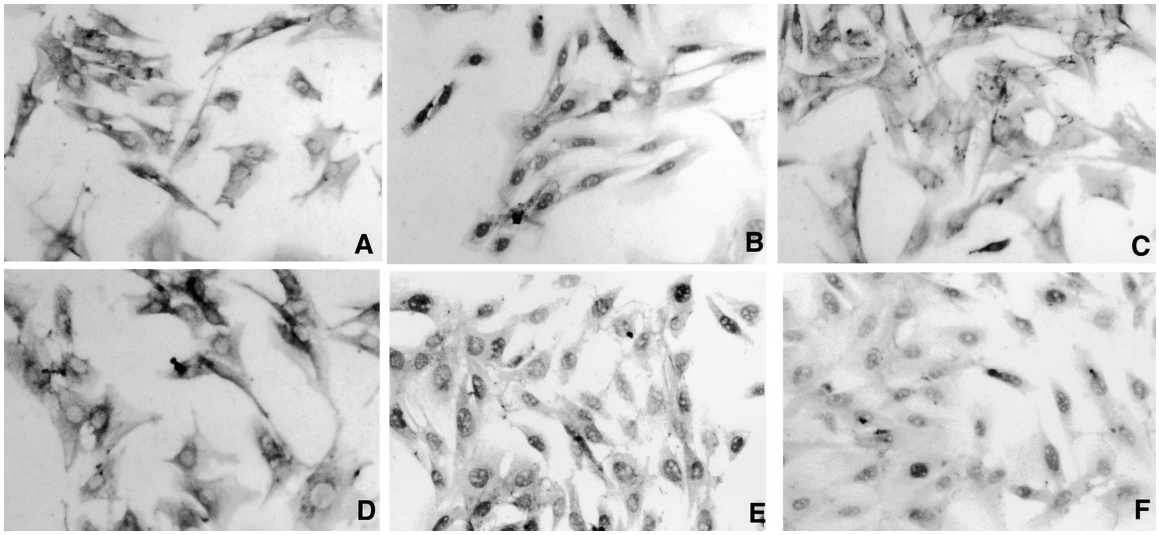

Fig. 8. Immunostaining of nuclear translocated p65/NF-κB induced by TNF-α: Effects of MG132, curcumin, PD98059, and calphostin C. (*A*) Control. (*B*) Incubation of MCs with TNF-α (5 ng/mL) for 15 min induced nuclear translocation of p65/NF-κB. (*C* and *D*) curcumin (40 μmol/L) and MG132 (10 μmol/L) blocked translocation of p65/NF-κB induced by TNF-α. (*E* and *F*) PD98059 (40 μmol/L) and calphostin C (400 nmol/L) did not affect TNF-α–stimulated nuclear translocation of p65/NF-κB (original magnification ×320).

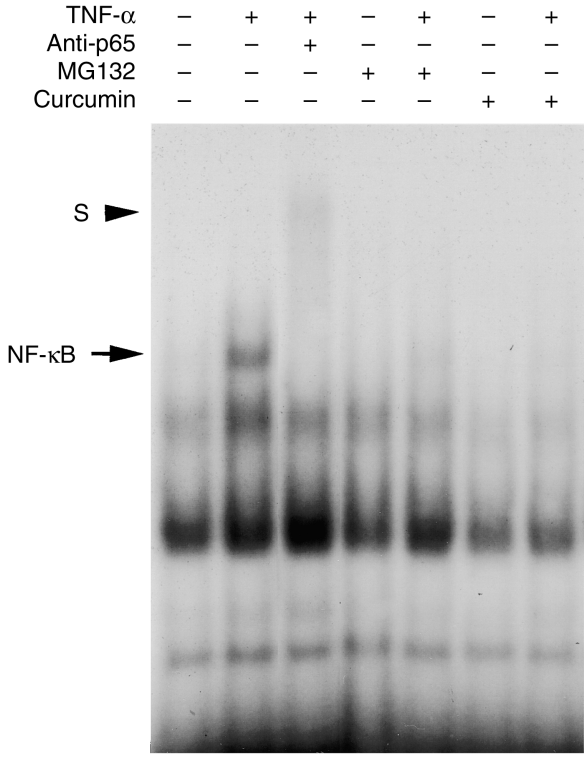

Fig. 9. Inhibition of TNF-α–induced NF-κB binding activity by MG132 and curcumin. MCs were treated with MG132 (10 μmol/L) or curcumin (40 μmol/L) for 1.5 hours prior to the addition of TNF-α for 1 hour. Nuclear extracts were probed for NF-κB. In lane 3, extracts were incubated with an antibody to the p65 subunit of NF-κB to verify the identify of the TNF-α–induced nuclear protein. The supershifted antibody-NF-κB complexes are indicated by the S arrowhead.

bound protein, which could be up-regulated by TNF-α in both time- and dose-dependent manners. Additionally, the conditioned media of TNF-α–activated MCs contained an approximately 75 kD soluble fractalkine that was likely shed from cell-bound fractalkine by metalloproteinase-dependent cleavage, as GM 6001 reduced the levels of soluble fractalkine in association with an increase in the amount of cell-bound fractalkine. This finding is consistent with recent observations that cleavage of cell-bound fractalkine, either by matrix metalloproteinases or TNF-α converting enzyme, is the major source for soluble fractalkine in cultured endothelial cells [9, 10]. Fractalkine was originally discovered from activated endothelial cells, and its ability to mediate both chemotaxis and adherence of mononuclear cells is unique among the chemokine superfamily [4]. However, the idea that fractalkine has chemotactic effects is not generally accepted [38]. Recent in vitro studies suggest that endothelial cell-derived fractalkine plays a role primarily in leukocyte adhesion rather than chemotaxis [39, 40]. In the kidney, it also is possible that soluble fractalkine generated on the surface of endothelium would be washed away under high blood flow conditions such as the renal glomerulus. In contrast, MCs are mesenchymal cells largely precluded from circulating blood flow. Thus, soluble fractalkine generated by MCs may bind to extracellular matrices and form local concentration gradients to attract leukocytes. This assumption is supported, at least partially, by the present in vitro chemotaxis assay showing that the TNF-α–activated MC conditioned media increased monocyte transmigration that could be attenuated, dose-dependently, by a neutralizing anti-fractalkine antibody. Together these data suggest that MC-derived soluble fractalkine is a novel signal for chemoattraction in the kidney, which may complement the action of endothelial cell-derived cell-bound fractalkine in the recruitment of CX_3CR1-bearing leukocytes.

Relatively little is known about the mechanism by which TNF-α stimulates fractalkine production by MCs. The intracellular signaling cascades initiated by TNF-α are complex, including those that activate protein kinases such as PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK, and transcription factors such as NF-κB and AP-1 [28, 30]. In this study, we showed that TNF-α–stimulated MC fractalkine mRNA and protein expression was attenuated by selective inhibitors of PKC (calphostin C) and p42/44 MAPK kinase (PD98059), but not PKA (H-89), PI 3-K (wortmannin), or p38 MAPK (SB203580). These results suggest that the cellular signals mediating TNF-α–stimulated fractalkine expression involve activation of PKC and p42/44 MAPK, rather than PKA, PI 3-K, or p38 MAPK pathways.

Further evidence supporting that PKC-related mechanism is operating in MC fractalkine gene expression is based on the results that calphostin C suppressed PMA-stimulated fractalkine mRNA expression, and the activation of PKC-depleted MCs with PMA did not stimulate MC fractalkine mRNA expression. These findings speak for the regulatory role of classical and/or novel isotypes of PKC in phobol ester-sensitive fractalkine expression in MCs. However, the depletion of (classical/novel) PKC activity by PMA failed to prevent MC fractalkine gene expression elicited by TNF-α, and the degree of TNF-α–stimulated fractalkine mRNA expression in PMA-pretreated MCs was comparable to that in non-PMA-pretreated cells. These observations raise the possibility that atypical PKCs mediate most, if not all, TNF-α–stimulated, PKC-dependent fractalkine gene expression in MCs.

Nuclear factor-κB and AP-1 are key nuclear factors regulating the transactivation of an array of genes involved in inflammation, cell proliferation, and apoptosis [29, 41, 42]. The present study has demonstrated that inhibitors of NF-κB (MG132, curcumin, quercetin, TPCK, and PDTC) abolished TNF-α–stimulated fractalkine mRNA and protein expression in MCs, indicating that NF-κB plays a crucial role in TNF-α–stimulated MC fractalkine expression. Our finding is in accord with a recent study finding that fractalkine induction by inflammatory cytokines is

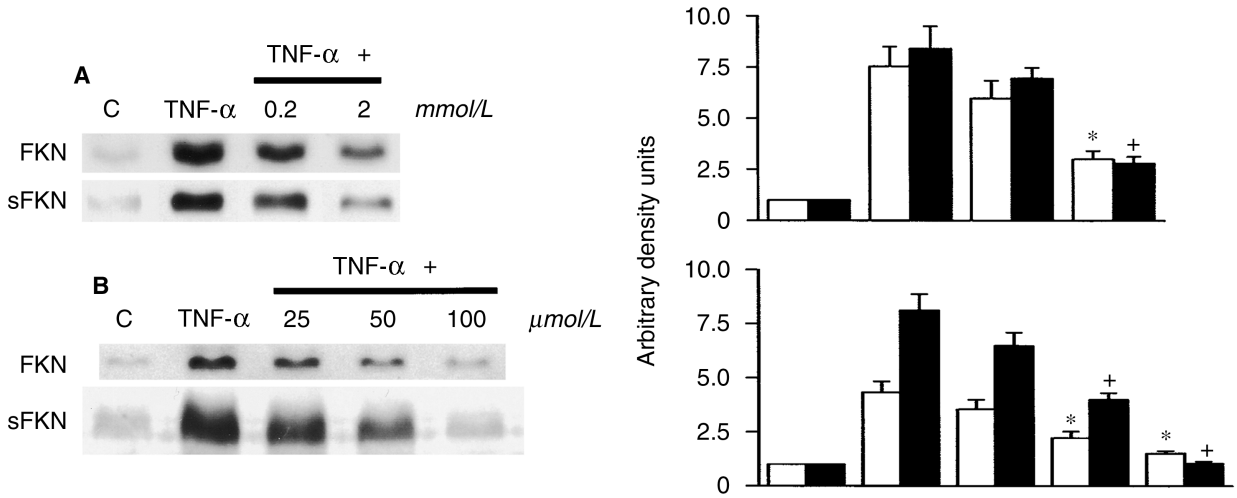

Fig. 10. Effects of cAMP-elevating agents on TNF-α–stimulated fractalkine mRNA and soluble fractalkine expression. MCs were incubated with TNF-α (5 ng/mL) for 4 or 24 h, with or without pretreatment with (*A*) db-cAMP (0.2 to 2 mmol/L), or (*B*) forskolin (25 to 100 μmol/L) for 15 and 45 minutes, respectively. Right panels show corresponding quantitative results of fractalkine/GAPDH mRNA ratios (□) and sFKN (■) relative to that of control. Values are mean ± SEM of four experiments. * and + denote $P < 0.05$ versus TNF-α–treated cells.

NF-κB–dependent in rat aortic endothelial cells [27]. Further, we found that the AP-1 inhibitor, curcumin, partially suppressed TNF-α–stimulated MC fractalkine expression at a concentration (20 μmol/L) that attenuated phospho-c-Jun levels but not nuclear translocation of p65/NF-κB. This suggests that TNF-α also stimulates fractalkine expression through the c-Jun/AP-1 pathway, independent of NF-κB activation. However, our data that MG132 completely blocked TNF-α–stimulated MC fractalkine expression in the presence of augmented levels of phospho-c-Jun argues against such an idea. We surmise that activation of c-Jun/AP-1 is necessary but not sufficient for TNF-α–stimulated fractalkine expression. In agreement with this notion, AP-1 proteins have been shown to interact with p65/NF-κB and augment its transactivation activity induced by TNF-α [43], and blockade of AP-1 activation by curcumin markedly inhibits the ability of TNF-α to activate κB-dependent gene expression at concentrations that do not inhibit activation of NF-κB [44].

In our experimental conditions, PD98059 and calphostin C were found to exert differential effects on TNF-α–activated signaling pathways and synergistically inhibit TNF-α–stimulated fractalkine mRNA and protein expression. We found that PD98059, a selective inhibitor of p42/44 MAPK kinase, inhibited TNF-α–stimulated fractalkine expression at a concentration that attenuated phospho-c-Jun but not phospho-PKCζ/ι levels, or nuclear translocation of p65/NF-κB. This suggests that mechanisms underlying the inhibitory effect of PD98059 is independent of the atypical PKC pathway and lies beyond p65/NF-κB nuclear translocation. Among those, AP-1 proteins have been shown to mediate TNF-α–activated gene expression via direct interaction with p65/NF-κB [43]. Thus, PD98059 may act by altering the c-Jun/AP-1 activity to modulate κB-dependent fractalkine gene transcription induced by TNF-α. In contrast, calphostin C inhibited TNF-α–stimulated fractalkine expression at a concentration that did not affect phosphorylation of p42/44 MAPK, c-Jun or translocation of p65/NF-κB. Calphostin C has been shown to inhibit the atypical PKC activity induced by IL-1β in MCs [45], and activation of PKC ζ has been reported to up-regulate κB-dependent transcription activity via direct phosphorylation of transactivation domain of p65/NF-κB [46]. Accordingly, calphostin C may act by blocking TNF-α–activated atypical PKC to down-regulate κB-dependent fractalkine gene expression. Our results that calphostin C decreased TNF-α–stimulated phospho-PKCζ/ι levels were in accord with such a notion. We surmise that TNF-α–activated p42/44 MAPK and PKC pathways are parallel signals that converge in κB-dependent fractalkine gene expression. A schematic diagram for the signaling pathways mediating TNF-α–activated fractalkine expression in cultured MCs is illustrated in Figure 11.

The present study showed that the cAMP-elevating agents, db-cAMP and forskolin, suppressed TNF-α–stimulated fractalkine mRNA and soluble protein expression, preceded by attenuation of phospho-p42/44 MAPK, phospho-c-Jun, but not phospho-PKCζ/ι levels, activated by TNF-α. The anti-phospho-p42/44 MAPK effect of these agents closely mimicked the action of PD98059. Indeed, induction of PKA activity by cAMP has been shown to block growth factor-induced *raf*-1 activity in many cell types, including the MCs [47, 48]. It is conceivable that these cAMP-elevating agents may act through antago-

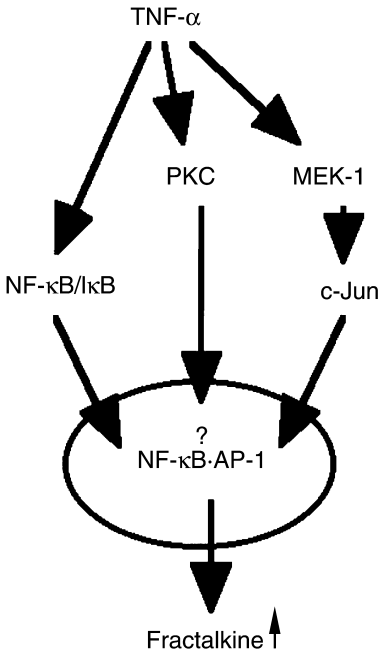

Fig. 11. Regulation of TNF-α–mediated fractalkine expression in MCs by NF-κB and c-Jun/AP-1 pathways. MEK-1 is p42/44 mitogen-activated protein kinase kinase.

nizing TNF-α–activated *raf*-1/p42/44 MAPK cascade to inhibit fractalkine expression. Additionally, increased intracellular cAMP levels have been shown to decrease IL-1β- or TNF-α–induced NF-κB activation in MCs using electrophoretic mobility shift analysis [49, 50]. However, our immunocytochemical staining failed to demonstrate that these cAMP-elevating agents could prevent TNF-α–induced nuclear translocation of NF-κB/p65. Since these agents did inhibit TNF-α–stimulated fractalkine expression, they may act at a step beyond NF-κB/p65 nuclear translocation. A possible mechanism could be modulating cofactors required for NF-κB transactivation. For example, the ability of db-cAMP to suppress TNF-α–activated c-Jun phosphorylation suggests that these agents may act, similar to PD98059, on the c-Jun/AP-1 pathway to down-regulate TNF-α–stimulated fractalkine expression. In view of the emerging importance of fractalkine in the regulation of adhesion and migration processes [7, 8, 11], modulation of fractalkine synthesis by cAMP-elevating agents may provide a means to suppress cellular recruitment in a variety of glomerular inflammatory disorders.

In summary, we have demonstrated that TNF-α dose-dependently stimulates both cell-bound and cleaved soluble fractalkine production by cultured rat MCs, and the soluble fractalkine mediates at least partially TNF-α–induced monocyte transmigration in vitro. TNF-α activation of PKCζ/ι, p42/44 MAPK, c-Jun/AP-1, and p65/NF-κB are involved in TNF-α–stimulated MC fractalkine expression. Uncoupling of p42/44 MAPK or c-Jun/AP-1 signals may contribute to cAMP inhibition of MC fractalkine production stimulated by TNF-α.

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